Immunology 1986 **58** 323–327

Bile immunoglobulin of the duck (*Anas platyrhynchos*)

I. PRELIMINARY CHARACTERIZATION AND ONTOGENY

PAMELA L. K. NG & D. A. HIGGINS *Department of Pathology, University of Hong Kong, Queen Mary Hospital, Hong Kong*

Accepted for publication 17 January 1986

SUMMARY

Immunoglobulin (Ig) of a single class was found in duck (*Anas platyrhynchos*) bile. Its molecular weight was 890,000; serum IgM was 800,000. Heavy chains were 75,000 for bile Ig, 86,000 for IgM, 67,000 for 7·8S IgG and 37,000 for 5·7S IgG. Antigenic comparison showed that bile Ig resembled IgM but carried additional determinants. The ontogeny of bile Ig was distinct from that of serum IgM and IgG. Thus, duck bile Ig appears to be an IgM-like molecule secreted independently of serum Ig.

INTRODUCTION

Ducks (*Anas platyrhynchos*), like their phylogenetically close relatives geese (*Branta canadensis*) and turtles (*Pseudamys scripta, Chelydra serpentina*), have three types of serum immunoglobulin (Ig): a 19S macromolecule resembling mammalian IgM, and two molecular sizes of IgG (Kubo, Zimmerman & Grey, 1973). The larger IgG has a sedimentation coefficient of 7·8S, and resembles chicken IgG, sometimes referred to as IgY to emphasize its primary structural differences from mammalian IgG (Leslie & Clem, 1969). The smaller is 5·7S and is antigenically identical to the 7·8S molecule minus a substantial portion of the Fc terminal of the heavy chain (Grey, 1967a; Zimmerman, Shalatin & Grey, 1971). The two forms of IgG are synthesized independently, i.e. the 5·7S molecule is neither a precursor nor a breakdown product of the 7·8S molecule (Grey, 1967b).

There have been no reported attempts to purify and characterize duck secretory Igs. Ducks are highly susceptible to a number of epitheliotrophic viruses and are believed to act as reservoirs of others. The existence of a secretory immune system in these animals and its response to mucosal infection would be important observations. We therefore examined duck bile as a source of putative secretory Igs.

MATERIALS AND METHODS

Ducks

Local outbred (Cantonese) and imported hybrid (White Pekin) ducks were used. Blood was collected from market ducks by jugular venesection at slaughter and from laboratory ducks by cardiac puncture. Bile was collected immediately after slaughter. In order to facilitate purification of Igs from sera of laboratory ducks, levels were first enhanced by repeated intravenous inoculation of bovine serum albumin or heterologous erythrocytes (Grey, 1967a). Sera and biles were stored at −20°.

Abbreviations: AGP, agar gel precipitin test; IE, immunoelectrophoresis; Ig, immunoglobulin; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RID, radial immunodiffusion.

Correspondence: Dr D. A. Higgins, Dept. of Pathology, Queen Mary Hospital, Hong Kong.

Salt precipitation of Igs

After extensive dialysis of serum or bile against 0·1 M phosphate-buffered saline, pH 7·2 (PBS), Igs were precipitated by the addition of crystalline anhydrous Na_2SO_4 to a final concentration of 14% w/v. After mixing for 2 hr at room temperature, the precipitate was recovered by centrifugation (5000 *g* for 30 min at room temperature) and redissolved in PBS. Precipitation was repeated three times.

Agar gel double diffusion (AGP)

AGP was done in thin layers of 1·5% Noble agar (Difco, Detroit, MI) in barbital buffer, pH 8·6, T/2 = 0·05, poured on microscope slides, and samples were applied to Perspex microtemplates set on the agar (Munoz, 1971).

Microimmunoelectrophoresis (IE)

IE was done in 1·5% Noble agar in barbital buffer, pH 8·6, T/2 = 0·05, poured on microscope slides (Scheidegger, 1955) held in immunoframes (Gelman, Ann Arbor, MI). Constant current was applied at 10 mA/immunoframe for 1 hr.

Gel filtration chromatography

Gel filtration was done by upward flow through columns of Sephadex G-200 superfine, Sephacryl S-300 or Sepharose CL-4B (Pharmacia, Uppsala, Sweden) equilibrated in 0·1 M Tris-HCl, pH 8·0, containing 1 M NaCl and 1 mM NaEDTA. Preparative chromatography was through columns 100 cm long × 2·5 cm diameter, and analytical chromatography

through columns 100 cm × 1·6 cm (Pharmacia). Sample volume was 1% of the column volume. Analytical columns were standardized using a range of proteins of known molecular weight (Pharmacia).

Polyacrylamide gel electrophoresis (PAGE)

PAGE was done according to Laemmli (1970), with modifications. Proteins were denatured by treating with 0·3 M 2-mercaptoethanol for 5 min at 90°, and this was followed by electrophoresis through stacking gels of 3% acrylamide in 0·125 M Tris-HCl, pH 6·8, and running gels of 12% acrylamide in 0·375 M Tris-HCl, pH 8·8. Electrode buffer was 0·025 M Tris, 0·192 M glycine, pH 8·3. All gels and buffers included 0·1% sodium dodecyl sulphate. Electrophoresis through the stacking gel was at 50 volts and through running gels was 120 volts. Molecular weights of protein bands were determined by comparison against a range of standards (Pharmacia).

Purification of duck serum Igs

IgM, 7·8S IgG and 5·7S IgG were purified from duck serum by salt precipitation followed by gel filtration through Sephadex G-200 superfine. The peaks were collected, concentrated by dialysis against polyvinyl-pyrrolidone and recycled through Sephadex G-200 superfine. This was repeated until each peak gave a single line in AGP against rabbit antiserum to whole duck serum. Protein concentrations were determined by a modification of the Kjeldahl method (Chase & Williams, 1968).

Antiserum preparation

Antisera were made in rabbits by inoculating protein solutions (serum, bile, salt-precipitated Igs, purified Igs) or immunoprecipitates prepared from IE, by methods described previously (Higgins, 1976).

Immunoadsorbents

Immunoadsorbents were prepared by cross-linking with glutaraldehyde (Avrameas & Ternynck, 1969) and used as described previously (Higgins, 1976).

Radial immunodiffusion (RID)

The method of Fahey & McKelvey (1965) was followed, with modifications. Antisera mixed in 1·5% Noble agar (Difco) in barbital buffer, pH 8·6, T/2 = 0·05, were poured in uniform thickness on glass plates. Samples were applied to wells, 1·5 mm in diameter, each plate having 22 sample wells and three for standards. Plates were incubated for 1 week immersed in paraffin oil. Diameters of precipitin rings were measured, and immunoglobulin concentrations of the samples were calculated by regression analysis against $\log_{10}$ concentration versus diameter of the standards.

RESULTS

IE of adult duck serum against RAD (rabbit anti-duck) serum and RAD-Igs (anti-Na_2SO_4-precipitated serum Igs) produced precipitin lines compatible with previous descriptions (Toth & Norcross, 1981) of duck IgM, 7·8S IgG and 5·7S IgG (Fig. 1). Against adult duck bile, RAD-serum gave two precipitin lines (Fig. 2). One migrated towards the anode and was morphologically and antigenically similar to serum albumin. The other, a heterogeneous line in the γ_1 region, was also precipitated by

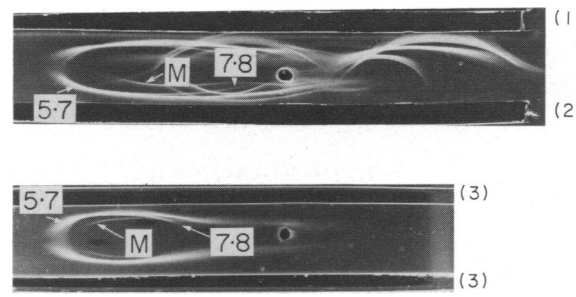

Figure 1. IE (cathode to the left) of duck serum against: (1) RAD-serum; (2) an early RAD-Igs, with some activity to proteins other than Igs; (3) a RAD-Igs reacting only against Igs. The lower configuration clearly demonstrates the morphology of duck serum IgM (M), 5·7S IgG (5·7) and the 7·8S 'spur' (7·8).

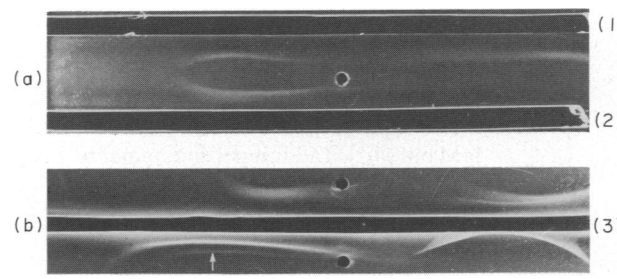

Figure 2. IE (cathode to the left) of: (a) duck bile; (b) upper well, duck bile; lower well, duck serum against: (1) RAD-serum; (2) RAD-Igs; (3) RAD-bile. The cathodal migrating arc in bile is Ig. RAD-bile precipitated serum IgM (arrow) inside an arc of 5·7S IgG, but no 7·8S spur.

RAD-Igs (Fig. 2). When RAD-bile was used in IE against bile the two lines seen with RAD-serum appeared, while against serum RAD-bile produced precipitin lines compatible with serum albumin, 5·7S IgG and IgM, but not 7·8S IgG (Fig. 2). Rabbit antisera to the bile γ_1 protein (soluble or immunoprecipitate) precipitated 5·7S IgG and IgM in IE of serum. If antisera to IgM, bile and bile Ig were absorbed with egg yolk (predominantly 7·8S IgG), they subsequently reacted only against IgM in serum and the bile Ig (Fig. 3a and b). Absorption of RAD-7·8S-IgG or RAD-5·7S-IgG with egg yolk removed all antibodies. However, absorption with bile made RAD-7·8S-IgG and RAD-5·7S-IgG class-specific (Fig. 3c and d) but removed all antibodies from RAD-IgM. The single precipitin lines produced in IE of serum by yolk-absorbed antisera to IgM and bile Ig merged in identity (Fig. 4a). The precipitin lines formed against bile met diffusely, not permitting antigenic comparison; however, the line produced by absorbed RAD-IgM was finer and closer to the antiserum trough, suggesting that it recognized fewer antigenic determinants than absorbed RAD-bile-Ig (Fig. 4b).

Yolk-absorbed RAD-bile-Ig was used in AGP to monitor the elution of Ig from gel filtration of bile. From Sephadex G-200, Ig occurred in the void volume along with bile pigment. To obtain pure bile Ig, it was first precipitated with Na_2SO_4 and, after extensive dialysis, the redissolved precipitate was filtered through Sephadex G-200. Fractions containing Ig were concentrated and recycled through Sephadex G-200, giving a single peak. This was, in turn, concentrated and filtered through Sepharose CL-4B; two peaks were obtained, the second contain-

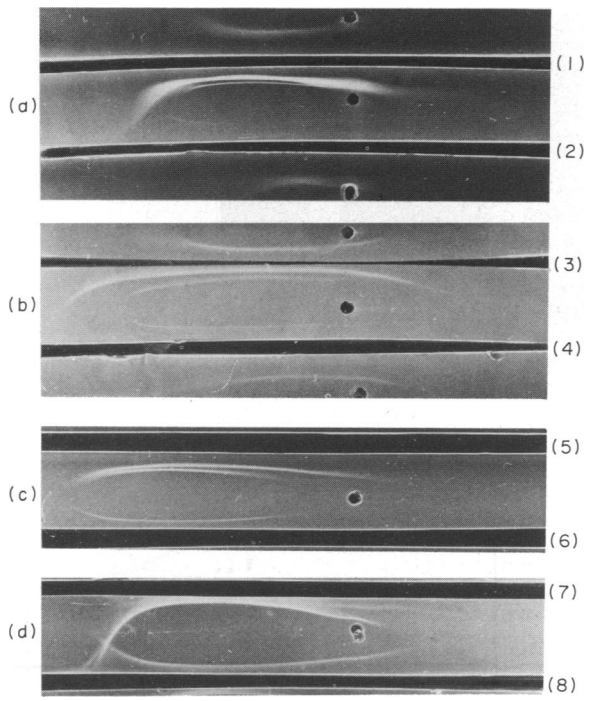

Figure 3. IE (cathode to the left) of duck serum (centre wells) and bile (outer wells in a and b) against: (1) RAD-IgM; (2) RAD-IgM absorbed with egg yolk; (3) RAD-bile-Ig; (4) RAD-bile-Ig absorbed with egg yolk; (5) RAD-7·8S-IgG; (6) RAD-7·8S-IgG absorbed with bile; (7) RAD-5·7S-IgG; (8) RAD-5·7S-IgG absorbed with bile.

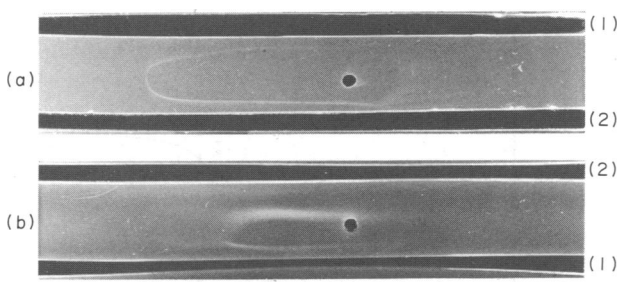

Figure. 4. IE (cathode to the left) of: (a) duck serum; (b) duck bile. Antisera: (1) RAD-IgM absorbed with egg yolk; (2) RAD-bile-Ig absorbed with egg yolk.

ing Ig. After concentration, this material reacted strongly against yolk-absorbed RAD-bile-Ig in AGP, and gave only a single line against RAD-bile. In AGP against yolk-absorbed RAD-bile-Ig the bile Ig precipitin line spurred over that of IgM (Fig. 5). However, yolk-absorbed RAD-IgM gave bile Ig and IgM lines which merged in identity (Fig. 5).

The molecular weights of duck immunoglobulins determined by gel filtration are given in Table 1 and the results of PAGE of degraded molecules are shown in Fig. 6. The results for 7·8S and 5·7S IgG confirmed previous reports (Zimmerman *et al.*, 1971). Intact IgM was 800,000 and bile Ig was 890,000 MW. Heavy chains were 75,000 MW for bile Ig, 86,000 for IgM, 67,000 for 7·8S IgG and 37,000 for 5·7S IgG. All classes possessed two sizes of light chains, estimated at 22,000 and 26,000 MW. There was no evidence in PAGE of additional polypeptide chains in bile Ig to explain its unique antigenicity or to account for its molecular weight being higher than that of IgM.

Biles and sera were collected from groups of two to seven healthy unvaccinated ducklings every few days between hatching and 72 days of age. Igs were quantified by RID using yolk-absorbed RAD-bile-Ig and RAD-IgM, and bile-absorbed RAD-7·8S-IgG. Serum IgM, serum IgG and bile Ig had distinct patterns of ontogeny (Fig. 7). Serum IgM was detected in low concentrations in 1-day-old ducklings. Levels increased to 1–4 mg/ml by 20–25 days and remained stable thereafter. IgG occurred at 2·7–7·6 mg/ml in 1- and 2-day-old ducklings, decreased to about 1 mg/ml by 10 days of age, then progressively increased to about 2 mg/ml during the remainder of the experiment. Bile Ig was first detected in one of the two birds examined at 26 days of age, and thereafter was observed in all birds examined. Between 38 and 50 days of age the bile Ig levels rose sharply, attaining concentrations of 1–10 mg/ml. There was considerable variation in the concentration of bile Ig, possibly reflecting differences in consistency between samples.

DISCUSSION

By conventional criteria (high molecular weight; immunoelectrophoretic heterogeneity in the gamma region; precipitated by sera containing antibodies to light chains; stimulated the production of rabbit antibodies to heavy and light chains), the molecule detected in and purified from duck bile was an Ig. Antigenic comparisons and molecular weight determinations on the intact molecule and its heavy chains indicated similarity with IgM, though bile Ig possessed additional antigenic determinants not occurring on IgM or recognized by rabbit antisera to IgM. Based on double-antibody radioimmunoassay inhibition employing carp and rabbit antisera to heavy chains of chicken 7S serum Ig, chicken bile Ig, human IgA and mouse IgA, other workers (Ambrosius & Hädge, 1983; Hädge, 1983; Hädge & Ambrosius, 1983, 1984) showed that: (i) duck and chicken IgY were antigenically closely related; (ii) chicken IgY was antigenically related to mammalian IgA; (iii) chicken bile Ig was unrelated to mammalian IgA. It was proposed that avian species possess serum Igs resembling IgM and IgA (the latter currently referred to as IgG or IgY) and a unique bile Ig tentatively called IgB (Ambrosius & Hädge, 1983; Hädge & Ambrosius, 1984). Two pieces of that data appear to invalidate the generalizations made: (i) no relationship was apparent between duck and chicken bile Igs (Fig. 8 in Hädge & Ambrosius, 1983); (ii) chicken IgY failed to inhibit binding between mouse IgA and rabbit antiserum to mouse α-chains (Fig. 9 in Hädge & Ambrosius, 1983). The capacity of duck bile Ig to bind to antisera against IgM heavy chains was not reported.

One important possibility is that duck bile Ig and serum IgM were cross-reacting through antigenic determinants other than heavy chains; as both are probably polymers, a J-chain-like polypeptide can be expected. This could explain why antiserum to bile Ig recognized common antigenic determinants on serum IgM and bile Ig, yet unique antigens only on the bile component. However, comparison of yolk-absorbed antisera to bile Ig and IgM in IE of duck serum produced IgM lines which merged. If these sera had recognized different heavy chains and had only antibodies to J-chain in common, then the line developed with

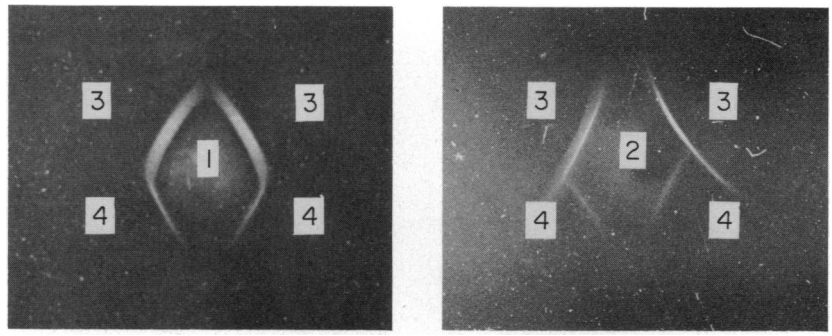

Figure 5. AGP comparison of the activity of yolk-absorbed RAD-IgM (1) and RAD-bile-Ig (2) against purified bile Ig (3) and serum IgM (4). The absorbed RAD-bile-Ig recognized determinants on bile Ig not present on IgM or recognized by the absorbed RAD-IgM.

Table 1. Molecular weight determinations by gel filtration on duck immunoglobulins

	Molecular weight ($\times 10^{-3}$)			
Gel	Bile Ig	IgM	7·8S IgG	5·7S IgG
Sephadex G-200 superfine	*	*	240	145
Sephacryl S-300	*	1100†	233	118
Sepharose CL-4B	890	800	‡	‡

* Protein eluted in void volume—molecular weight determination not possible.

† Determination at the upper limit for the technique—probably inaccurate.

‡ Protein too small for accurate determination of molecular weight by this method.

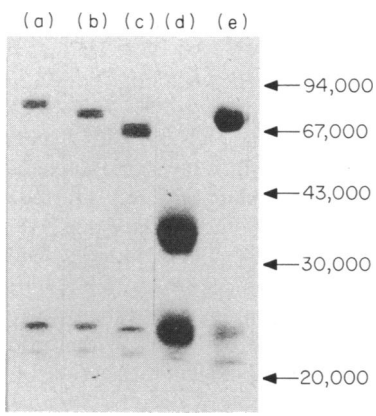

Figure 6. PAGE of 2-ME-treated duck IgM (a), bile Ig (b and e, different concentrations), 7·8S IgG (c) and 5·7S IgG (d). Positions of standards of known MW are indicated.

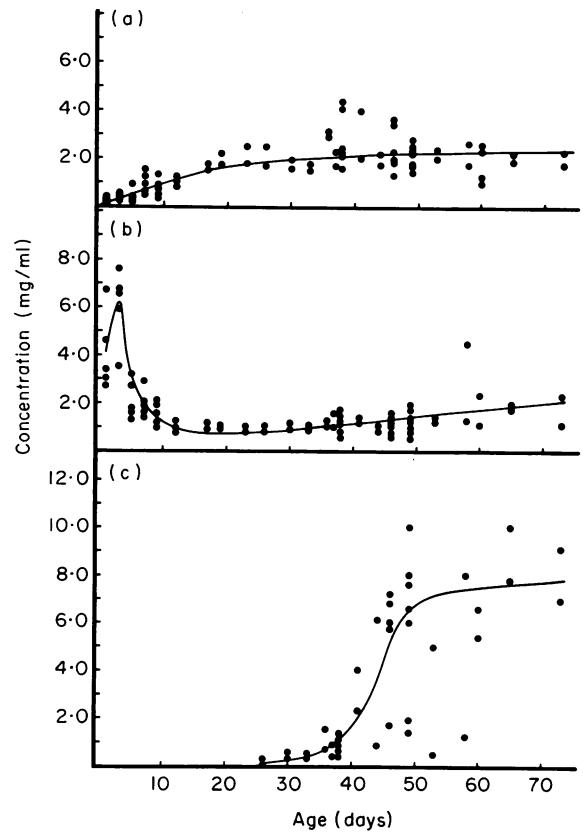

Figure 7. Ontogeny of immunoglobulins in ducks 1–72 days old: (a) serum IgM; (b) serum IgG; (c) bile Ig. Each point represents an individual bird; curves have been fitted to indicate trends.

RAD-IgM should have formed a spur over the line developed with RAD-bile-Ig. We conclude that both sera recognized μ-chains. The nature of the additional antigenic determinants on bile-Ig requires clarification.

Proof that duck bile Ig is a secretory immunological mechanism will require several observations.

(i) Ideally, the molecule should possess an antigenic or functional homologue of secretory component. In the chicken, there is disagreement whether secretory component does (Parry & Porter, 1978; Peppard, Rose & Hesketh, 1983) or does not (Watanabe & Kobayashi, 1974) occur on bile IgA, though its occurrence on IgA in intestinal secretions is generally accepted. Molecular characterization and comparison of duck serum IgM and bile Ig is continuing in this laboratory.

(ii) Production of the molecule should be independent of the production, presence or concentration of serum Igs. The ontogeny and concentrations of duck serum Igs resembled those

reported for chickens (Van Meter, Good & Cooper, 1969; Leslie & Martin, 1973; Higgins & Calnek, 1975). The 5·7S and 7·8S IgGs were not studied separately because of their considerable antigenic similarity, but IE of the duckling sera (data not shown) indicated no temporal correlation between the presence of either subclass and the appearance of bile Ig. The ontogeny of bile Ig was clearly distinct from that of the serum Igs, supporting the concept that this molecule is an independent mucosal system. However, this result does not eliminate the possibility that the distinct ontogeny of bile Ig reflects an essential, but late developing, mechanism for hepatic transport of IgM.

(iii) Antibody activity of the molecule and immunological memory for the system should be independent of the serum Ig response (Lemaitre-Coelho, Jackson & Vaerman, 1978b; Andrew & Hall, 1982a, b). Other studies (Higgins *et al.*, unpublished observations) have revealed that 4 weeks after infection of ducklings with influenza A viruses, bile Ig and serum IgG, but not serum IgM, possess haemagglutination-inhibiting antibody activity. Thus, the IgM-like bile Ig is unlikely to be derived from serum IgM.

The secretory immune system has received greatest attention in chickens and mammals in which the presence of IgA emphasizes the distinction of this system from serum antibody. Prior to the evolution of IgA, animals must have had some form of mucosal protection. Cells of intestinal mucosa of *Xenopus laevis* produce a molecule similar to, yet distinct from, IgM (Hsu, Flajnik & Du Pasquier, 1985). Our studies of duck bile indicate that secretory Ig in this species is an IgM-like yet independent molecule. In mammals, IgM has retained its capacity for being transported by the liver from serum to bile (Lemaitre-Coelho, Jackson & Vaerman, 1978a; Peppard, Jackson & Hall, 1983), while the increased production of IgM at mucosal surfaces of mammals with IgA deficiency (Thompson, 1970) might be a reflection of ancestral capabilities.

ACKNOWLEDGMENTS

This work was supported by grants from the University of Hong Kong, the Pauline Chan Medical Research Fund and the Wing Lung Bank Medical Research Fund. It was also greatly assisted by the generosity of duck farmers in the New Territories, and of colleagues in the Departments of Biochemistry and Microbiology, and the Clinical Biochemistry and Haematology Units of this University, the photographic assistance of Mr S. K. Lau and the secretarial skills of Miss W. Mo.

REFERENCES

AMBROSIUS M. & HÄDGE D. (1983) Phylogeny of low molecular weight immunoglobulins. *Dev. Comp. Immunol.* **7,** 721.

ANDREW L. & HALL J.G. (1982a) IgA antibodies in the bile of rats. I. Some characteristics of the primary response. *Immunology*, **45,** 169.

ANDREW L. & HALL J.G. (1982b) IgA antibodies in the bile of rats. II. Evidence for immunological memory in secretory immunity. *Immunology*, **45,** 177.

AVRAMEAS S. & TERNYNCK T. (1969) The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. *Immunochemistry*, **6,** 53.

CHASE M.W. & WILLIAMS C.A. (1968) Chemical analyses. In: *Methods in Immunology and Immunochemistry* (eds M. W. Chase and C. A. Williams), Vol. II, Chapter 12. Academic Press, New York.

FAHEY J.L. & MCKELVEY E. M. (1965) Quantitative determination of serum immunoglobulins in antibody-agar plates. *J. Immunol.* **94,** 84.

GREY H.M. (1967a) Duck immunoglobulins. I. Structural studies on a 5·7S and 7·8S γ-globulin. *J. Immunol.* **98,** 811.

GREY H.M. (1976b) Duck immunoglobulins. II. Biological and immunochemical studies. *J. Immunol.* **98,** 820.

HÄDGE D. (1983) Immunochemical studies on the so-called chicken IgA. *Dev. Comp. Immunol.* **7,** 725.

HÄDGE D. & AMBROSIUS H. (1983) Evolution of low molecular weight immunoglobulins. III. The immunoglobulin of chicken bile—not an IgA. *Molec. Immunol.* **20,** 597.

HÄDGE D. & AMBROSIUS H. (1984) Evolution of low molecular weight immunoglobulin. IV. IgY-like immunoglobulins of birds, reptiles and amphibians, precursors of IgA. *Molec. Immunol.* **21,** 699.

HIGGINS D.A. (1976) Fractionation of fowl immunoglobulins. *Res. vet. Sci.* **21,** 94.

HIGGINS D.A. & CALNEK B.W. (1975) Fowl immunoglobulins: quantitation in birds genetically resistant and susceptible to Marek's disease. *Infect. Immun.* **12,** 360.

HSU E., FLAJNIK M.F. & DU PASQUIER L. (1985) A third immunoglobulin class in amphibians. *J. Immunol.* **135,** 1998.

KUBO R.T., ZIMMERMAN B. & GREY H.M. (1973) Phylogeny of immunoglobulins. In: *The Antigens* (ed. M. Sela), Vol. I, Chapter 6. Academic Press, New York.

LAEMMLI U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*, **277,** 680.

LEMAITRE-COELHO I., JACKSON G.D.F. & VAERMAN J.-P. (1978a) High levels of secretory IgA and free secretory component in the serum of rats with bile duct obstruction. *J. exp. Med.* **147,** 934.

LEMAITRE-COELHO I., JACKSON G.D.F. & VAERMAN J.-P. (1978b) Relevance of biliary IgA antibodies in rat intestinal immunity. *Scand. J. Immunol.* **8,** 459.

LESLIE G.A. & CLEM L.W. (1969) Phylogeny of immunoglobulin structure and function. III.Immunoglobulins of the chicken. *J. exp. Med.* **130,** 1337.

LESLIE G.A. & MARTIN L.N. (1973) Modulation of immunoglobulin ontogeny in the chicken: effect of purified antibody specific for μ chain on IgM, IgY and IgA production. *J. Immunol.* **110,** 959.

MUNOZ J. (1971) Double diffusion in plates. In: *Methods in Immunology and Immunochemistry* (eds C. A. Williams and M. W. Chase), Vol. III, p. 146. Academic Press, New York.

PARRY S.H. & PORTER P. (1978) Characterization and localization of secretory component in the chicken. *Immunology*, **34,** 471.

PEPPARD J.V., JACKSON L.E. & HALL J.G. (1983) The occurrence of secretory IgM in the bile of rats. *Clin. exp. Immunol.* **53,** 623.

PEPPARD J.V., ROSE M.E. & HESKETH P. (1983) A functional homologue of mammalian secretory component exists in chickens. *Eur. J. Immunol.* **13,** 566.

SCHEIDEGGER J.-J. (1955) Une micro-méthode de l'immune-électrophorèse. *Int. Archs Allergy appl. Immunol.* **7,** 103.

THOMPSON R.A. (1970) Secretory piece linked to IgM in individuals deficient in IgA. *Nature (Lond.)*, **226,** 946.

TOTH T.E. & NORCROSS N.L. (1981) Immunoelectrophoresis of duck sera and immunoglobulins. *Avian Dis.* **25,** 1.

VAN METER R., GOOD R.A. & COOPER M.D. (1969) Ontogeny of circulating immunoglobulins in normal, bursectomized and irradiated chickens. *J. Immunol.* **102,** 370.

WATANABE H. & KOBAYASHI K. (1974) Peculiar secretory IgA system identified in chickens. *J. Immunol.* **113,** 1405.

ZIMMERMAN B., SHALATIN N. & GREY H.M. (1971) Structural studies on the duck 5·7S and 7·8S immunoglobulins. *Biochemistry*, **10,** 482.